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IMMUNOASSAY USING HAPTEN–ENZYME CONJUGATES

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1. Introduction

Recently, we described an "enzyme-immunoassay" for human chorionic gonadotrophin (HCG *), using the enzyme horse radish peroxidase (HRP) as a label for the antigen instead of radioactive iodine, as used in radioimmunoassay [1]. Independently, Engvall et al. developed similar assay systems with alkaline phosphatase-labelled antigen [2, 3] or antibody [4].

We have now applied the enzyme-immunoassay principle to the determination of haptens **. We used oestradiol and oestriol, coupled through succinyl bridges to HRP, and antibodies against these steroid hormones. The resulting assay systems are more or less analogous to the radioimmunoassays for steroid hormones [5] that have been developed after the initial demonstration that steroid hormones can act as haptens [6, 7].

2. Materials and methods

2.1. *Reagents*

Oestradiol-17β-hemisuccinate and oestriol-16α, 17β-dihemisuccinate were prepared by heating the oestrogens with excess succinic anhydride in pyridine, followed by hydrolysis of the 3-ester with dilute sulphuric acid and by repeated crystallisation from methanol. HRP, grade IV, R.Z. 0.6, was purchased from Miles-Seravac; BSA, Cohn fraction V, from Armour; sec. butylchloroformate and tri-*n*-butylamine from Fluka; 5-aminosalicylic acid from EGA-Chemie. Various oestrogen sulphates and glucuronides were bought from Sigma. Lactalbumin was from De Meyerij, Veghel, The Netherlands. Dasp anti-rabbit (sheep antibodies against rabbit γ-globulin, covalently linked to cellulose) is an Organon product.

2.2. *Preparation of oestrogen–protein conjugates*

E–BSA and E–HRP were prepared by a mixed anhydride reaction, using sec. butylchloroformate. E–BSA was prepared essentially according to Erlanger et al. [8], using a molar ratio E/BSA of 60. The number of E-residues per BSA molecule in the conjugates was estimated, both spectrophotometrically and by amino group determination with trinitrobenzene sulphonic acid [9], to be about 25 for E_2–BSA and 32 for E_3–BSA. E–HRP conjugates were prepared using E/HRP ratios ranging from 5 to 40 (see Results). After the reaction they were passed over a Sephadex G-50 column, dialyzed and lyophilized, before subjecting them to further purification procedures (see Results).

2.3. *Preparation of antisera against E–BSA*

Rabbits received 3 × 1.25 mg immunogen subcutaneously in complete Freund's adjuvant, with 14-day intervals, followed by an intravenous injection of 1.25 mg immunogen in physiological saline. The rabbits were bled one week after the booster injection. If necessary, the animals received a second series of injections.

* Abbreviations used are: HCG: human chorionic gonadotrophin; HRP: horse radish peroxidase; E: oestrogen; E_1: oestrone; E_2: oestradiol; E_3: oestriol; BSA: bovine serum albumin.

** Dutch patent application 7016396, filed 10th November 1970.

2.4. *Enzyme-immunoassay procedures*

The double antibody solid phase method was used, employing Dasp anti-rabbit (sheep antibodies against rabbit γ-globulin, covalently linked to cellulose) [10, 11]. Incubation times and buffer were the same as in the Dasp enzyme-immunoassay for HCG [1], except for the phosphate concentration of the buffer, which was increased to 0.1 M in order to avoid non-specific adsorption of E–HRP conjugates to the immunosorbent.

2.5. *Peroxidase assay*

HRP-activity was determined with 5-aminosalicylic acid as oxygen-acceptor, as described earlier [1].

3. Results

E–HRP conjugates were prepared while using molar E/HRP ratios ranging from 5 to 40. Fig. 1 shows the relation between E/HRP ratio and "immune reactivity", defined as the percentage of the enzyme activity that can be bound by excess antibody against the corresponding steroid, for E_2–HRP and E_3–HRP. Since virtually no enzyme activity is lost during the coupling reaction, the yield of each conjugate before purification equals its immune reactivity.

An E_2–HRP and an E_3–HRP, both prepared with an E/HRP ratio of 20, were subjected to various purification procedures. Iso-electric focusing and chromatography on Sephadex LH-20 gave no fractions with increased immune reactivity. Table 1 shows the results of gel filtration on Sephadex G-75 and Biogel P-60, and of centrifugation on a linear 20–60% sucrose gradient at 283,000 *g* for 40 hr. Since centrifugation gave the products with the highest immune reactivity, the following experiments were performed with conjugates, purified by that method.

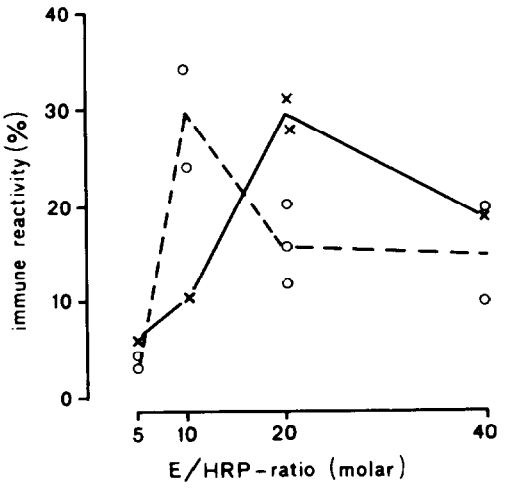

Fig. 1. Effect of E/HRP ratio (molar) during coupling on the immune reactivity of the unpurified E–HRP conjugates. (x——x——x)E_2–HRP; (o----o----o)E_3–HRP.

Table 1
Purification of E–HRP conjugates by various methods.

Purification step	E_2–HRP		E_3–HRP	
	Immune reactivity (%)	Yield * (%)	Immune reactivity (%)	Yield * (%)
Non-purified	28	28	20	20
Sephadex G-75	48	10	34	14
BioGel P-60	41	6	27	8
Density gradient ultracentrifugation	64	5	95 **	0.2 **
			56 **	0.8 **

* Yield is expressed as: Immune reactivity product × $\frac{\text{enzyme activity product}}{\text{initial enzyme activity}}$.

** The E_3–HRP ultracentrifuge fractions were pooled in two portions with different immune reactivity. The data apply to the pooled fractions with approximately the highest immune reactivity.

Fig. 2 and 3 show the displacement of E–HRP conjugates from their binding to antibodies by E_1, E_2 and E_3. Fig. 2 shows the curves obtained with an anti-E_3 serum combined with an E_2–HRP and an E_3–HRP, fig. 3 shows the curves for the same conjugates and an anti-E_2 serum. We obtained similar results with various other antisera, although no antiserum showed as large a difference in reaction with E_2–HRP and E_3–HRP as the one shown in fig. 3. With appropriate combinations of conjugate and antiserum, nanogram amounts of hapten could be detected.

In order to evaluate the specificity of the assay systems, we tested various compounds, including oestrogen sulphates and glucuronides and non-oestrogenic steroids, with two different conjugate–antiserum

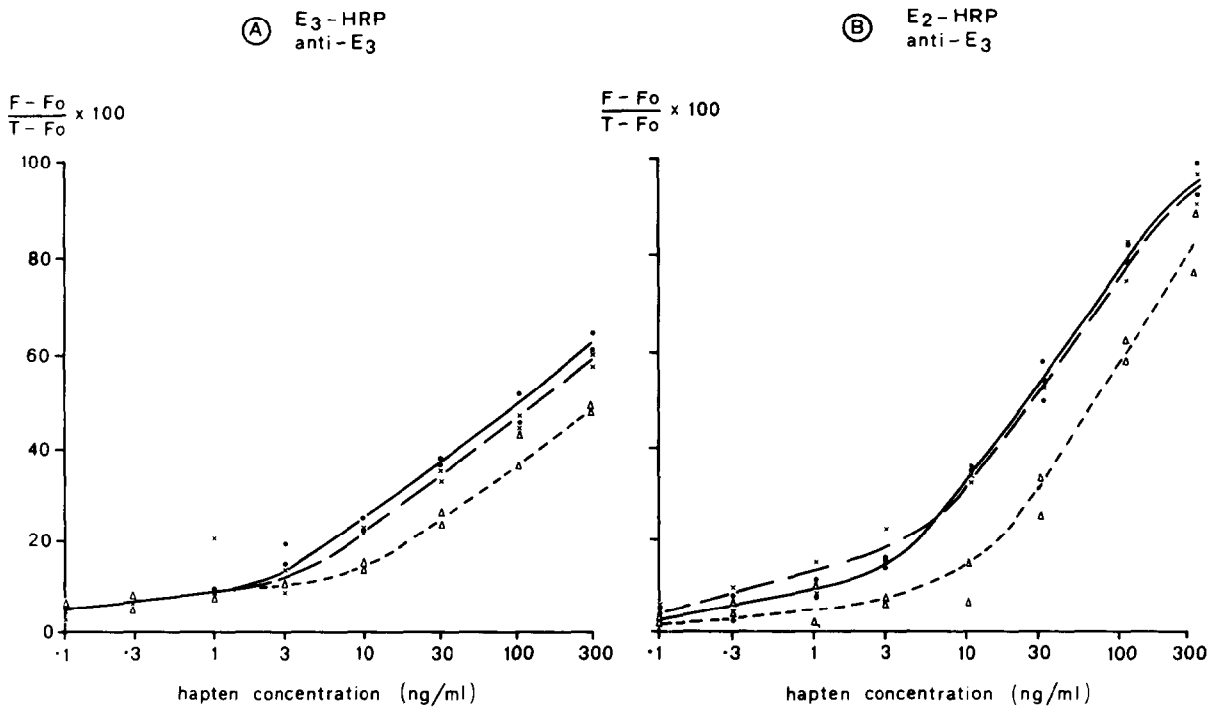

Fig. 2. Oestrogen assay systems: An E_3–HRP (A) and an E_2–HRP (B), with anti-E_3 serum 405 in dilutions of 1/1400 and 1/1200, respectively. (•——•——•)E_1; (x— —x— —x)E_2; (△---△---△)E_3. F = activity in supernatant with added hapten. F_0 = activity in supernatant without added hapten. T = total enzyme activity. This amount of enzyme gives an A_{450}^{1cm} of about 0.400 in the enzyme assay.

combinations. The results are shown in table 2. Compounds showing lack of parallelism with the arbitrary standard oestrone are not strictly comparable with the other compounds. Therefore, they are marked in the table.

4. Discussion

A high quality of E–HRP conjugates is essential for the sensitivity of the resulting oestrogen enzyme-immunoassays. The immune reactivity is the only criterion we have used for the selection of the conjugates. Possibly, more sensitive assay systems can be developed if the conjugates requiring the smallest amount of antiserum to bind for example 50% of the enzyme activity or the conjugates giving the steepest antiserum dilution curves are selected.

The systems in figs. 2 and 3 are not strictly comparable because of the different antiserum dilutions used and the differing properties of E_2–HRP and E_3–HRP conjugates. It appears, however, that more sensitive assay systems arise when E_2–HRP is combined with an anti-E_3 serum, and vice versa, than in homologous systems. This "mismatch" does not seem to affect the specificity of the systems. Cross-reaction between oestrogens is as high as could be expected from experience with radioimmunoassays. Cross-reaction with non-oestrogen steroids is very low.

The highly effective displacement obtained by E–hemisuccinates, as compared to the unmodified oestrogens (table 2), suggests that the antibodies are directed against both the oestrogen molecule and the succinyl bridge or parts of it. The finding that E_2–17-monoacetate and E_2–17-monopropionate displace ^{3}H-E_2 more efficiently from E_2–17-succinyl-BSA antibodies than E_2 itself [12], supports this assumption. This affinity for succinyl bridges may affect the sensitivity of our test system, in which the enzyme-labelled hapten contains succinyl bridges, and the haptens to be

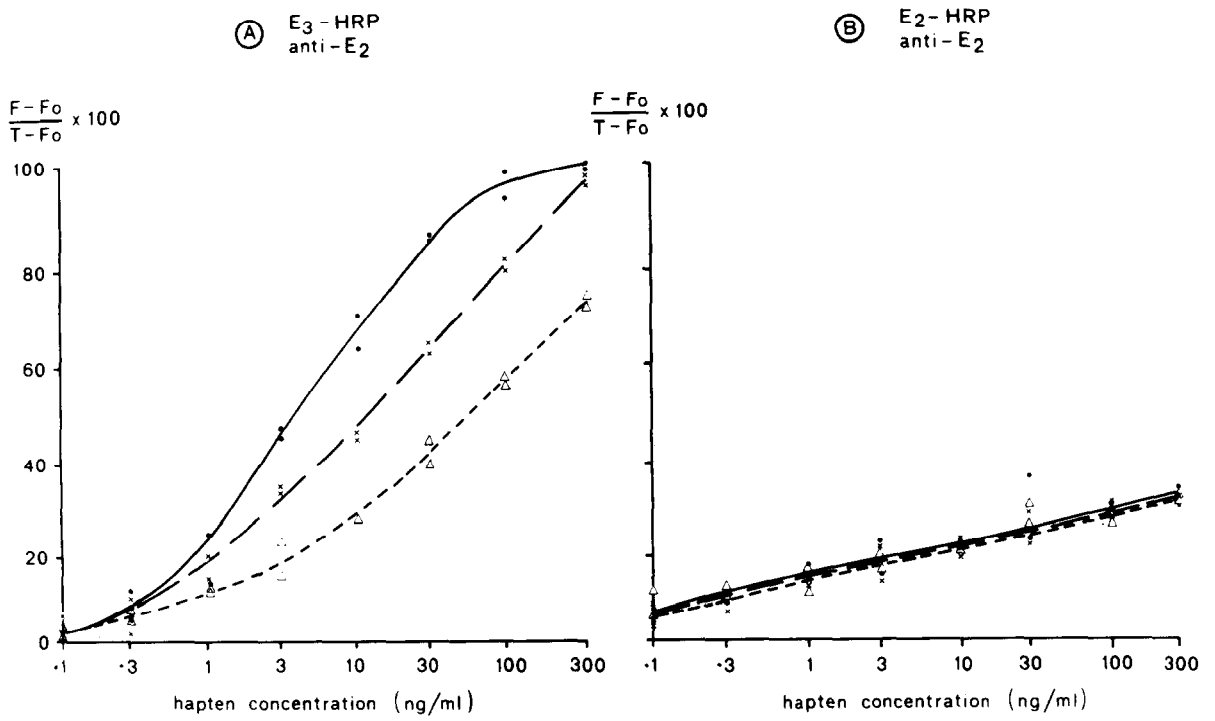

Fig. 3. Oestrogen assay systems: An E_3–HRP (A) and an E_2–HRP (B), with anti-E_2 serum 408 in dilutions of 1/700 and 1/1000, respectively. For explanation of the symbols see legend to fig. 2.

determined do not. The same effect may be expected in radioimmunoassays employing ^{125}I-labelled E–succinyl-BSA [13, 14], thus reducing the theoretical sensitivity gain from the higher specific activity.

Oestrogens conjugated with sulphate or glucuronide at site 3 do not react in our systems, whereas 16- or 17-glucuronide oestrogens do, in many cases even as well as the unconjugated oestrogen (table 2). This might be expected, since Midgley reported that structural differences at the end distal to conjugation have the greatest influence on the cross-reaction, while alterations near the site of conjugation have little effect [14]. Our E_2–HRP/anti E_3 system, which crossreacts completely with E_3–16- and E_3–17-glucuronide, is potentially applicable to E determination in pregnancy urine, where most of the E occurs as 16- or 17-conjugated E_3. The application of a radioimmunoassay using anti-E_3 for E determination in pregnancy urine has been described [15].

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Table 2
Cross-reaction† of various steroids in two oestrogen assay systems (means of two or three determinations).

Hapten	E_3–HRP anti-E_2 408	E_2–HRP anti-E_3 405
Oestrone	100	100
Oestrone-3-sulphate	0.3	< 0.1
Oestrone-3-glucuronide	< 0.1	< 0.1
Oestradiol	70	110
Oestradiol-17-hemisuccinate	330 *	740
Oestradiol-3-glucuronide	1	1
Oestradiol-17-glucuronide	100	86
Oestriol	17	36
Oestriol-16,17-dihemisuccinate	22	1750 *
Oestriol-3-sulphate	< 0.1	< 0.1
Oestriol-3-glucuronide	< 0.1	< 0.1
Oestriol-16-glucuronide	3 **	32 **
Oestriol-17-glucuronide	7	49
16-Epi oestriol	20	55
17-Epi oestriol	8	27
Testosterone	< 0.03	< 0.03
Progesterone	0.1	< 0.03
Cortisone	< 0.03	< 0.03
Corticosterone	< 0.03	< 0.03

† The cross-reaction is expressed as $\frac{\text{amount of oestrone that displaces 50\% of enzyme activity}}{\text{amount of hapten that displaces 50\% of enzyme activity}} \times 100$

* Lack of parallelism with oestrone: steeper curve.

** Lack of parallelism with oestrone: shallower curve.

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